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(54) Title: SKIN COMPOSITES (57) Abstract The invention herein described relates to a method to prepare tissue, typically but not exclusively, skin for use in the repair of wounds and/or damaged tissue and/or cosmetic reconstruction.		

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SKIN COMPOSITES

The invention relates to a method to prepare skin tissue for use in, particularly
5 but not exclusively, the repair of wounds and/or damaged tissue and/or
cosmetic reconstruction.

Skin is a highly complex organ covering the external surface of the body.
Skin functions, amongst other things, to prevent water loss from the body and
10 to act as a protective barrier against the action of physical, chemical or
infectious agents. Skin has an elastic property and varies in thickness from 0.5
mm, on for example the eyelids, to 4 mm on for example the palms and soles.

Skin is composed of two layers. The outer layer, which is comparatively thin
15 is called the epidermis. It is several cells thick and has an external layer of
dead cells that are constantly shed from the surface and replaced from below
by a basal layer of cells, called the stratum germinativum. The epidermis is
composed predominantly of keratinocytes which make up over 95% of the cell
population, the rest include dendritic cells such as Langerhans and pigmented
20 cells called melanocytes. It is essentially cellular and non vascular, there
being relatively little extra cellular matrix except for the layer of collagen and
other proteins beneath the basal layer of keratinocytes. Keratinocytes of the
basal layer are constantly dividing, and daughter cells subsequently move
outwards, during which they undergo a period of differentiation and are
25 eventually sloughed off from the surface.

The inner layer of the skin is called the dermis and is composed of a network
of collagenous extracellular material, elastic fibres, blood vessels and nerves.
Contained within it are hair follicles and associated sebaceous glands,

collectively known as the pilosebaceous unit. The interface between the epidermis and dermis is extremely irregular and consists of a succession of papillae, or finger like projections.

- 5 Skin is therefore a highly complex organ the cells of which are periodically replaced. Although skin provides a hard wearing and resilient outer surface skin can be physically stressed with relative ease thus leading to irreparable damage. Damage of this sort can result from: high impact contusions, diseases or burns.

10

Diseases that result in irreparable damage include gastric ulcers; ischemic and/or arterial ulcers; and/or diabetic ulcers.

- Of these, so called ambulatory ulcers are the most common and result from
15 poor venous blood supply to the lower limbs. Venous insufficiency results in severe necrosis leading to deep open wounds and scarring. The scarring results in a condition referred to as lipodermatosclerosis which is a hardened and leathery skin overlying sites of fibrin cuffs. This is particularly evident in diabetes sufferers where foot ulcers can result in amputation of toes and/or
20 feet. There are a number of treatments of diabetes that alleviate many of these symptoms. However the ulcers can be deep and do not heal well. In these situations skin grafts may be appropriate to remedy the condition.

- Gastric ulcers are a common ailment of the major industrialised nations. There
25 is a strong correlation between the presence of *Helicobacter pylori* and the incidence of gastric ulcers and this correlation has changed how patients prone to gastric ulcers are treated. However in those individuals suffering from established gastric ulcers the only remedial action is to surgically repair the

stomach wall. This may require replacing the tissue with an autograft from a region of the stomach that is unaffected.

In addition to the above, skin is particularly susceptible to heat damage which can be of varying severity. For example, first degree burns affect the outer layer of the skin, causing pain redness and swelling. Second degree burns affect both the outer and underlying layer of skin causing pain, redness, swelling and blistering. Third degree burns extend into deeper tissue causing brown or blackened skin that may result in irreparable damage; this requires surgery to remove dead skin and replacement, via grafting of skin, to repair the wound. Currently three types of graft are available to a surgeon:

- i) an autograft, in which a piece of tissue is removed from one area of a patient's body and placed in another location;
- ii) an allograft, in which a section of tissue from one human, for example a cadaver, is grafted onto another human; and
- iii) a xenograft, where tissue is harvested from another animal species, for example a pig, and placed over the wound area.

Autografts can be problematic due to the availability of suitable tissue and the added trauma to the patient during the removal of the tissue from another part of the body to the wound area. Allografts are limited by immunological reactivity of the host and the availability of donor tissue. Xenografts are even more problematic due to the severe immunological reactivity.

Currently a number of approaches are being used to address the problems identified above. One approach is to provide human skin composites or

reconstructed skin that have reduced immune reactivity leading to a decrease in tissue rejection by the patient. Alternatively, research has been directed to the development of artificial skin composites which provide many of the features of natural skin. In yet a further alternative strategy the combination of
5 cultured epithelium in combination with either an allograft dermis or synthetic dermis have been used to decrease rejection.

Culturing epithelial cells by the technique described by Green *et al* (PNAS USA 1979; 76: 5565 – 5568 (35)) made it possible to prepare large numbers of
10 epithelial sheets reliably within about four weeks post burn. These epithelial sheets are used in isolation of other replacement tissue. Typically epidermal grafts tend to be fragile leading to late graft loss, so that long term “take” rates are often much lower than initial take rates. The results of early and late graft take were reported to be improved by the technique of excising the burn tissue,
15 covering it with an allograft, allowing the allograft to take, followed by shaving off the epidermal layer and replacing it with autogenous cultured epithelial autograft sheets (Cuono *et al* PNAS, 1987; 80; 826 – 835, (37)). However problems still remain with this technique and include unreliable allograft take, early rejection, infection, necrosis of the allograft, and difficulty
20 in shaving the allograft to the required depth.

One method of avoiding these problems is to prepare epidermal-dermal composites *in vitro*, before application to the burn wound. This approach has been explored, and it has been demonstrated that epidermal-dermal composites
25 can be successfully prepared *in vitro*. However, there are several issues that need to be addressed before these techniques become clinically applicable on a routine basis. It is important that the allogenic dermal tissue used should be sterile before clinical use to eliminate the risk of transmission of disease. The donor tissue should have a minimum of potentially antigenic allogeneic

cellular material. Both the sterilisation procedures and methodology for removal of donor cells could affect the nature of the allograft tissue. It is therefore very important to provide a collagen based matrix which retains many structural features of human skin. This will facilitate the infiltration of the host keratinocytes and accessory cells, to reconstitute skin at the wound site and to provide a resilient and visually appealing skin graft.

It is important that the allograft is free of the donor's host cells, thereby reducing immunogenicity, and is free from infectious agents that may compromise skin graft acceptance by the patient. Currently methods of sterilisation and removal of cellular elements from allogeneic skin result in disruption of the collagen matrix leading to reduced colonisation of the graft by the hosts keratinocytes and early graft rejection.

It is therefore an object of the invention to provide a method for producing a skin composite that is both sterile and retains its natural conformation; and the skin composite produced by the method.

It is a further object of the invention to provide a skin composite that is both functionally and aesthetically equivalent to natural skin for use in surgical repair of damaged tissue and cosmetic applications.

According to a first aspect of the invention there is provided a method for providing a skin composite comprising;

- i) harvesting a collagen based tissue from a human or animal;
- ii) removing the cellular elements from said collagen based tissue;

- iii) dehydrating said collagen based tissue in the presence of a sterilising agent; and
- iv) removing said dehydrated and sterilised collagen based tissue from said
5 dehydrating and sterilising agents.

We have found that the above methodology and in particular step (iii) thereof provides a skin composite that is unique in that it is both sterile and importantly, it retains its integrity or natural conformation.

10

In a preferred embodiment of the invention said collagen based tissue is derived from skin, preferably human skin and more preferably still is an allograft.

- 15 It will be apparent that the collagen based tissue may originate from alternate sources, for example and not by way of limitation, gastric tissue, venous/arterial vessels, heart valves, bone, oral mucosa. In addition these alternative tissues may serve as autografts, allografts, or xenografts.

- 20 It will be apparent from the above description that an allograft is the preferred source of collagen based tissue. This reduces the physical and psychological stress on the patient as graft material may be provided from a skin bank and abrogates the need to remove skin from a region of the patient's body as would be required if the graft was an autograft.

25

It will be apparent to one skilled in the art that methods exist to treat collagen based tissues to remove cellular elements from said tissue. For example and not by way of limitation, dermal samples derived from skin can be incubated long term in phosphate buffered saline or hyperosmotic saline solutions or

proteolytic enzymes (e.g. trypsin, dispase) or combinations thereof. Samples are monitored periodically to assess the cellularity of the sample until such time as the dermis is reduced to a collagen matrix which is devoid of cellular elements. Acellular dermal samples can then be stored at -20°C without appreciable loss of structure. As mentioned, it is desirable to provide a collagen matrix that is essentially free of cellular material to reduce graft rejection due to immune recognition by the patient.

In yet a further preferred method of the invention said dehydration of collagen based tissue is through incubation with a liquid dehydration agent.

In yet a further preferred method of the invention said liquid dehydration agent is glycerol.

In yet still a further preferred method of the invention said dehydration of collagen based tissue is through a sequential and incremental increase in glycerol concentration.

In yet still a further preferred method of the invention said glycerol concentration is increased from at least 50% glycerol to at least 98% glycerol.

In yet a further preferred method of the invention said increase in glycerol concentration is in increments of at least 10% glycerol.

In yet a further preferred method of the invention said dehydration agent is isopropanol

In yet still a further preferred method of the invention said dehydration of collagen based tissue is through the sequential and incremental increase in isopropanol concentration.

- 5 In yet still a further preferred method of the invention said isopropanol concentration is increased from at least 50% isopropanol to at least 98% isopropanol.

- 10 In yet a further preferred method of the invention said increase in isopropanol concentration is in increments of at least 10% isopropanol.

In yet still a further preferred method of the invention said sterilising agent is a fluid.

- 15 It will be apparent to one skilled in the art that the sterilising agent may be provided as either a liquid or gas.

In yet still a further preferred method of the invention said sterilising agent is a liquid.

20

In yet still a further preferred method of the invention said sterilising agent is gaseous ethylene oxide.

- 25 In yet a further preferred method of the invention said ethylene oxide is provided at at least 15% ethylene oxide, at least 85% carbon dioxide at an atmospheric pressure of at least 5.5 atmospheres at at least 55°C for at least 30 mins. Under these conditions a concentration of 1200 mg ethylene oxide per litre is maintained during the sterilising cycle.

It will be apparent to one skilled in the art that the combination of dehydration in the presence of a sterilising agent has a twofold beneficial effect on the collagen based tissue. Firstly, the dehydration step maintains the integrity of the tissue and facilitates its long term storage. Secondly, the severe effects of for example ethylene oxide, on the collagen based tissue is ameliorated by the presence of, for example glycerol but retains its sterilising effect on infectious agents.

According to a second aspect of the invention there is provided a skin composite produced by the aforescribed method.

According to a fourth aspect of the invention there is provided a therapeutic composition comprising the skin composite prepared by the method of the invention.

In a preferred embodiment of the invention said therapeutic composition is provided with storage means which facilitates and/or maintains the natural structural conformation of the skin composite.

According to a fifth aspect of the invention there is provided a method according to any previous aspect or embodiment of the invention for the preparation of collagen based tissues for use in cosmetic reconstructive surgery.

It will be apparent that for example burn victims, both require remedial tissue reconstruction to provide a functional skin replacement and cosmetic replacement to provide an aesthetically pleasing appearance to the grafted skin. In many examples it is also necessary to provide cosmetic surgery in an attempt to return the appearance of the damaged skin to its former state.

However the collagen based skin composites prepared by the method of the invention also has applications in cosmetic surgery, for example and not by way of limitation, to remove birth marks, skin blemishes or scars. Collagen based tissue composites prepared by the method of the invention would be
5 equally applicable to cosmetic uses as to major tissue reconstruction as a consequence of, for example third degree burns.

An embodiment of the invention will now be described, by example only, and with reference to the following figures and tables;

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Figure 1 represents collagen IV in normal skin (b) and residual collagen IV in glycerol sterilised de-epidermised dermis(d), ethylene oxide sterilised de-epidermised dermis (f) and glycerol pretreated ethylene oxide sterilised de-epidermised dermis (h);

15

Figure 2 represents the appearance of a skin composite prepared by glycerol treatment;

20

Figure 3 represents the appearance of a skin composite prepared by ethylene oxide treatment;

Figure 4 represents the appearance of a skin composite prepared by glycerol and ethylene oxide treatment;

25 Figure 5 represents the appearance of a skin composite prepared using keratinocytes expanded in culture and added either as a suspension or as an autograft sheet;

Table 1 represents the production of de-epidermised dermis using ethylene oxide and 1M NaCl under various conditions;

5 Table 2 represents the influence of sterilisation protocols on the physical properties of dermis;

Table 3 represents the scoring system used to assess skin composites;

10 Table 4 represents a comparison of the in vitro appearance of skin composites prepared using ethylene oxide and glycerol/ ethylene oxide sterilised skin;

Table 5 represents the influence of sterilisation methodology on the appearance of composites grafted onto nude mice;

15 Table 6 represents the influence of sterilisation methodology on the presence of collagen IV in composites on nude mice; and

20 Table 7 represents a comparison of the in vitro appearance of skin composites prepared using freshly isolated and cultured keratinocytes using ethylene oxide sterilised skin.

MATERIALS AND METHODS

Materials

25 Phosphate buffered saline (PBS) tablets were obtained from Oxoid, Unipath, Hampshire, UK. Dulbecco's modified Eagle's medium was from ICN Flow, Thame, Oxfordshire, UK. Ham's F12, glutamine, amphotericin B, penicillin and streptomycin were from Gibco Europe, Life Technologies, Paisley, UK. Foetal calf serum (FCS) was from Advanced Protein Products, Brierley Hill,

West Midlands, UK. Hydrocortisone was from Nova Biochem, Nottingham. Trypsin was from Difco Laboratories, Detroit, USA. Dispase and Collagenase A were from Boehringer-Mannheim, Lewes, East Sussex, UK. Glycerol and isopropanol were from BDH Laboratory Supplies, Lutterworth, Leicestershire, UK. MTT, cholera toxin, epidermal growth factor (EGF), adenine, insulin, sodium chloride, transferrin, triiodothyronine and primary antibody to laminin, transferrin, triiodothyronine, ethylenediaminetetra acetic acid (EDTA), trypan blue, anti-human laminin antibody, anti-human collagen IV antibody were from Sigma Chemical Co, Poole, Dorset. ABC immunostaining kits was bought from Vector Laboratories, Peterborough, UK. Primary antibody to collagen IV was obtained from DAKO Laboratories, High Wycombe. ; Stainless steel clips, Ligaclips were from Ethicon, Ltd, Edinburgh, UK. Stainless steel grids and rings were supplied by the Department of Medical Physics, Royal Hallamshire Hospital, Sheffield, UK. All pre-sterilised plastic containers for use in tissue culture were from Costar, High Wycombe Buckinghamshire, UK. All tissue was handled in class II laminar flow hoods, provided by Walker Safety Cabinets, Glossop, Derbyshire, UK. Nude mice were obtained and investigated in the Research Department of Northwick Park Hospital, Middlesex, UK. Silicone wound chambers of 1cm diameter used on the nude mice were purchased from Renner GMBH, Dannstadt, Germany.

Freshly isolated keratocytes

Human adult keratinocytes were obtained from harvesting of split thickness skin grafts (STSG) after routine plastic surgical procedures (breast reductions and abdominoplasty), following Goberdhan et al.'s (1993) (17) modification of the Rheinwald and Green (1975) (18) methodology. In brief, small pieces (~0.5cm²) of skin were incubated in 0.1% (w/v) trypsin solution for 16 hours at 4°C allowing the epidermal and dermal layers of individual skin samples to be separated and the cells between to be collected, washed, re-suspended and

counted. These cells were now ready for use as primary suspensions in skin equivalents, production of secondary keratinocytes and keratinocyte sheets.

Keratinocytes expanded in culture

- 5 Primary keratinocytes in suspension were seeded onto a pre-coated feeder layer of lethally irradiated 3T3 cells (i.3T3). These were cultured and refed every 3 days until approximately 80% confluent, usually by 7-9 days. Residual i.3T3 were removed with 0.02% EDTA before the secondary keratinocytes were detached using trypsin. The suspension of cells was centrifuged at 200g
10 for 5 minutes and the pellet of cells re-suspended in a known volume of keratinocyte medium (KM). A cell count was then performed and these cells were now ready for use as secondary suspensions of keratinocytes.

Keratinocyte Sheets

- 15 2×10^6 primary keratinocytes in suspension were seeded onto a petri dish that had been pre-coated with a feeder layer i3T3 cells (2×10^6 per petri dish) and cultured as previously described until a confluent multilayer sheet of cells. The cultured epithelial sheet was then detached by incubating for approximately 20-30 minutes in a 0.25% dispase solution. Once detached, a tegapore backing
20 dressing was applied to the sheet of cells and secured to the dressing using stainless steel ligaclips, so that the basal cells were facing up, on the backing dressing. The keratinocyte sheets were now ready for application.

Human Fibroblasts

- 25 Fibroblasts were obtained as described by Ralston et al. (In Press)(16). Briefly the dermis left after trypsinisation of split thickness skin graft for keratinocyte extraction was washed in PBS, finely minced and placed in 0.5% collagenase A at 37°C for 16 hours.

The resulting digest was collected by centrifugation at 200g for 10 minutes and re-suspended in fibroblast media (FM). At this point a cell count could be performed. This was then placed in T75cm² flasks and incubated under the standard culture conditions described.

5 Passaging of Human Fibroblasts

80-90% confluent fibroblasts were enzymatically detached using 0.1% w/v trypsin for 5-10 minutes. The detached cells were aspirated and the trypsin was neutralised by the addition of FCS or FM. The suspension of cells was centrifuged at 200g for 5 minutes. The pellet of cells was re-suspended in a
10 known volume of FM solution. A viable cell count was performed cells were now ready for use in composites, or for further passaging. Only cells between passage 4 and 9 were used in the composites.

Preparation of Dermal Substrates

15 Several stages were involved in preparation of dermis. These were sterilisation, de-epidermisation, acellularisation and finally preparation of the DED for experiments. All procedures were carried out using standard aseptic techniques.

20 Sterilization Techniques

The 2 sterilisation techniques used for the preparation of dermis were glycerol and ethylene oxide. We also explored pre-treatment of skin prior to ethylene oxide sterilisation by dehydrating skin using either glycerol or isopropanol.

25 Sterilization Using Glycerol

STSG was placed sequentially in an excess of 50%, then 85% glycerol in PBS for 24 hours each at room temperature. Finally the STSG was transferred into 98% glycerol, where it was stored at room temperature, for 6 weeks. This methodology enabled the slow dehydration of skin. Previous work from this

laboratory showed that 3 weeks in 98% glycerol at room temperature was sufficient to inactivate intracellular viruses in normal human fibroblasts (Marshall et al 1995)(19). The skin samples were ready to go on to the next stage, that of acellularisation.

5

Sterilization Using Ethylene Oxide

Skin was transferred from theatre to the laboratories in normal saline. The first step was to wash the skin in distilled water to eradicate the saline and thereby prevent any future chlorhydrin production during ethylene oxide sterilisation.

- 10 STSG were washed in 6 changes of and then immersed in fresh distilled water in a sealed container and placed in a shaking water bath for a minimum of 10 minutes. This was repeated a minimum of 3-4 times more. Excess water was allowed to drip off and then the skin was placed in a 90mm petri dish base with fenestrated aluminium foil on top and frozen to -20°C at $1^{\circ}\text{C}/\text{minute}$,
15 before being freeze-dried overnight. Freeze-dried skin was then double bagged before ethylene oxide sterilisation, using a standard SterivitTM process, using a mixture of 15% ethylene oxide and 85% carbon dioxide at a pressure of 5.5 atmospheres at 55°C . We also explored an alternative method of ethylene oxide sterilisation using a protocol which did not exceed 37°C . This produced
20 similar reticular damage to that observed with our own conditions.

- A minimum of 3 days was allowed for sample aeration to eradicate residual ethylene oxide gas. Confirmation of ethylene oxide sterilisation was by spore strip results, which took 3-5 days and samples were only released after these
25 results were obtained.

Once returned from CSSD, sterilised dry skin could be kept at room temperature in their vacuum-sealed packets, until required for use. This provided a convenient method of storing skin.

Dehydration of Skin prior to Ethylene Oxide Sterilization

In order to overcome some problems due to ethylene oxide damage to skin, we explored 2 methods of dehydrating the skin. These were pre-treatment with:

5

1. Glycerol

STSG were placed in a large volume of sterilised 50% glycerol in PBS followed by 85% glycerol in PBS, each for 4 hours at room temperature. Finally the STSG was transferred into 98% glycerol, and stored at room
10 temperature for 40 hours.

The skin was then firmly padded dry to remove any excess glycerol and placed in autoclave bags and seal with autoclave tape. These were then sent to CSSD for vacuum sealing of bags and ethylene oxide sterilisation as already
15 discussed above. Once returned from CSSD, skin can be kept at room temperature until required for use.

2. Isopropanol.

STSG were placed in a large volume of sterilised 50% isopropanol in PBS
20 followed by 75% isopropanol in PBS, each for 4 hours at room temperature. Finally the STSG was transferred into 98% isopropanol , and stored at room temperature for 40 hours.

The skin was then firmly padded dry to remove any excess glycerol and
25 placed in autoclave bags and seal with autoclave tape. These were then sent to CSSD for vacuum sealing of bags and ethylene oxide sterilisation as already discussed above. Once returned from CSSD, skin can be kept at room temperature until required for use.

De-epidermisation

Following previous work in this laboratory we used two methods for de-epidermisation. PBS was used for glycerol sterilised skin (Moore *et al* 1993)(17), whilst 1M sodium chloride was used for skin sterilised by ethylene oxide.

De-epidermisation of Glycerol Treated Skin

Glycerolised sterilized skin was rehydrated for at least 4 hours with several changes of sterile PBS containing 625µg/ml amphotericin B, 1000iu/ml penicillin and 1000µg/ml streptomycin. The graft was then placed in an excess of PBS for 4-14 days at 37°C to produce a dermal/epidermal split using the modified technique of Krejci *et al* (1991)(20). Such separation techniques have shown collagen IV and laminin retention on the dermal surface (Woodley *et al* 1983 and Moore *et al* 1993)(21,22).

Grafts were transferred into sterile 90mm petri dishes, the epidermis was gently peeled from the dermis and discarded. The remaining dermis was washed in PBS with 625 µg/ml amphotericin B, 1000iu/ml penicillin and 1000µg/ml streptomycin for the rest of the acellularisation duration.

De-epidermisation of Ethylene Oxide Treated Skin

All skin sterilised with ethylene oxide, irrespective of any prior preparation, underwent this process.

Freeze-dried sterilised skin was removed from the vacuum sealed packets and hydrated in a large volume of sterile PBS for 24 hours at 37°C to eradicate any residual ethylene glycol. Skin was then immersed in 1M sodium chloride solution for 6-8 hours at 37°C and the epidermis was removed using sterile forceps. Skin was then rehydrated in an excess of KM for 48 hours at 37°C.

Acellularisation

Following ethylene oxide sterilisation, the split thickness skin contained anucleate epithelium and no obvious fibroblasts in the dermis. After de-epidermisation the dermis was completely acellular and no further processing was required. For glycerolised skin, however, it was necessary to remove cells as below:

The de-epidermised dermis was washed in sterile PBS with 0.625µg/ml amphotericin B, 1000iu/ml penicillin and 1000µg/ml streptomycin and then immersed in PBS with 0.625 µg/ml amphotericin B, 1000iu/ml penicillin and 1000µg/ml streptomycin at 37°C. Twice weekly changes of PBS with 0.625 µg/ml amphotericin B, 1000iu/ml penicillin and 1000µg/ml streptomycin were performed.

After 6 weeks, biopsies of the dermis were taken for confirmation of acellularity by H&E stain. Once grafts were free of cells, a biopsy was taken for culture to confirm sterility. Once this was confirmed to be negative, the DED was ready for use in composite manufacture.

Assessment of Stress and Strain of Different Dermal Preparations

The stress-strain characteristics and tensile strength were measured by the method of Vogel (1971). Dumb-bell shaped skin strips of 12mm length and 4mm width were punched out in the same orientation from all the dermal preparations. These strips were immersed and stored in phosphate buffer solution (pH 7.2) at 4°C prior to testing. At room temperature, each wet strip was tested uniaxially in tension on an Instron Tensile Tester (Model 1112) in a liquid cell with the deformation applied along the strip length. The thickness of the samples was measured using a screw gauge. The broadened ends were

gripped between the Instron grips and the stress-strain curves were recorded at an extension rate of 0.5cm/min. The tensile strength (kg/cm^2) was obtained by dividing the alternate load by the area of original cross section (thickness X width).

5

Preparation of de-epidermised dermis (DED) for experiments

For each experiment, DED from a single donor was used. The DED was cut into approximately 2 x 2cm pieces for the preparation of skin composites. These pieces of DED were placed in FM in a sterile petri dish for 48 hours in a

10 37°C incubator to confirm sterility prior to culture with cells.

Preparation of De-Epidermised Dermis (DED) for Experiments

For each experiment, DED from a single donor was used. The DED was cut

15 into approximately 20 x 20mm pieces for the preparation of skin composites. These pieces of DED were placed in FM in a sterile petri dish for 48 hours in a 37°C incubator to confirm sterility prior to culture with cells.

Skin Composite Production

20 In a six well cell culture plate DED was placed with the reticular surface facing uppermost. A stainless steel ring with a 1cm internal diameter, was placed in the centre of the DED and pressed down with sterile forceps to effect a seal. A suspension of 1×10^5 fibroblasts in FM was placed in the ring and the surrounding DED was bathed in FM. The FM within the ring was replaced

25 after 24 hours with fresh media. After 48 hours incubation, the medium was aspirated from the well and the stainless steel ring was removed. The DED was turned so that the papillary surface was uppermost and the stainless steel ring was applied onto the corresponding papillary surface. A suspension of 1×10^6 primary keratinocytes (or 3×10^5 in the case of secondary keratinocytes

unless otherwise stated) in KM was applied in the stainless steel ring and the remainder of the well was filled with KCM. 24 hours later the KM within the ring was aspirated and the keratinocytes were re-fed fresh KM. The following day, 48 hours after application of keratinocytes, the KM was aspirated from
5 inside the rings and from the wells and the skin equivalents were raised onto stainless steel grids and fresh KM was added to the level of the base of the skin equivalent so that they were at an air liquid interface.

Skin composites were cultured for the required period, usually 10 days.
10 Medium was changed every 2 - 3 days and at the end of the time period, they were assessed for their histological morphology and basement membrane and some of these were assessed for their in-vivo performance on nude mice.

To analyze the contribution of the keratinocytes and fibroblasts, composites
15 were made using different combinations of cells at different seeding densities and different DED. These combinations included DED with no cells, keratinocytes alone and keratinocytes and fibroblasts together. If cells were not to be added at a particular step, then 0.5ml of the appropriate medium was added in the same way into the ring to avoid any variation in experimental
20 conditions.

Grafting of Nude Mice

Anaesthetised nude mice were placed on a sterile towel. Graft sites were prepared on the anterolateral back with routine aseptic preparation using 70%
25 alcohol and this was then washed off with sterile normal saline.

Two graft sites were applied, one on either side. The back was chosen, as it was inaccessible for the animal to preen this area. A small circular full thickness skin wound was made just big enough to allow the insertion of the

silicone wound chambers (Renner GMBH, Dannstadt, Germany) down to the panniculus carnosus, the vascular layer of fascia. The adjacent skin was dissected free of subcutaneous tissue to make room for the flanges of the silicone wound chamber. If the hole was made too large then it was tightened
5 up with a 6/0 prolene suture tied as a purse string. Care was taken to avoid haematoma formation as this can interfere with graft vascularisation. Once the wound chamber was in place, the skin equivalent was trimmed and applied as a lay-on graft. The skin equivalent was then dressed with an overlying non-adherent dressing and saline soaked damp gauze, which was cut to size to fill
10 the chamber. Once both sides had chambers and dressings in situ then 2.5cm Elastoplast™ was wrapped around the chambers and the trunk of the mouse tightly enough to secure the positions and not compromise the breathing of the mouse. The mice were then placed in individual cages for 2 weeks, after which they were sacrificed by overdose of intraperitoneal barbiturate. Once
15 the mouse was dead, the wound chambers were removed and the wound and its underlying base were excised *en bloc*. The specimen was cut into pieces for frozen section and light microscopy.

RESULTS

Production of sterile acellular allodermis suitable for clinical use

20 Our requirements were that skin should be sterilized and that this sterilization technique should give allodermis with physical properties resembling that of fresh dermis. This sterilization should allow removal of the epidermis on a relatively large area of skin readily and simultaneously provide good retention of basement membrane antigens and a cell-free allodermis from this material.

25

Hence, in the first step of this work, we examined the impact that the method of removal of the epidermis had on retention of basement membrane and loss of cells in ethylene oxide sterilized skin. This followed previous work from our laboratory (Ghosh et al 1997)(23) in which we showed that ethylene oxide

could be used to produce allodermis suitable for composite production although the process of ethylene oxide sterilization led to allodermis with varying degrees of dermal damage. Nevertheless ethylene oxide is a relatively rapid and well accepted method of sterilization. Hence, it was used in this study in preference to glycerol sterilization, which requires a minimum of 3 weeks.

Table 1 documents removal of epidermis using 1M sodium chloride at 4°C and 37°C. We compared removal of epidermis from fresh skin and from ethylene oxide sterilized skin varying the temperature and duration of exposure to 1M sodium chloride. Confirmation of the presence of basement membrane antigens was achieved by immunostaining for collagen IV. The results summarized in Table 1 show that at 37°C we were able to remove the epidermis reliably within 8 hours both from fresh skin and from ethylene oxide skin whereas it requires 72 hours at 4°C to obtain equivalent removal of the epidermis from fresh skin at 4°C. In the case of skin at 4°C, we only lost basement membrane after de-epidermisation at 48 hours.

To check for survival of viable fibroblasts, we attempted to culture fibroblasts at different stages of processing from the ethylene oxide sterilized split thickness skin. Up to 4 hours, there were anucleate cells and cellular debris in the ethylene oxide sterilized skin that had been treated with 1M sodium chloride at 37°C. By 8 hours, there was no evidence of any cellular material. We were unable to culture any viable fibroblasts from ethylene oxide sterilized dermis, which had been subjected to sodium chloride removal of epidermis at any timepoint.

Figure 1 illustrates the morphology of (a) normal skin, of (b) glycerol sterilized de-epidermised dermis, of (c) ethylene oxide sterilized de-

epidermised dermis and of (d) glycerol pre-treated ethylene oxide sterilized de-epidermised dermis. As can be seen, glycerol sterilization gives a dermis, which is indistinguishable from that of normal skin at the light microscopy level. In contrast, ethylene oxide sterilization often leads to some degree of damage to the reticular dermis. However, glycerol pre-treatment of ethylene oxide sterilized dermis gave a dermal architecture which was indistinguishable from that of normal skin.

Ethylene oxide sterilized and glycerol pre-treated ethylene oxide sterilized dermal preparations were routinely produced in 5 days and, by this time, were totally acellular.

Figure 2 illustrates the presence of residual collagen IV in (a) normal skin, (b) glycerol sterilized de-epidermised dermis, (c) ethylene oxide sterilized de-epidermised dermis and (d) glycerol pre-treated ethylene oxide sterilized de-epidermised dermis. As can be seen, there is good retention of collagen IV in the ethylene oxide treated dermis whether pre-treated with glycerol or not. This contrasts strongly with the extent of collagen IV retention in the glycerol sterilized dermis (which required soaking in PBS for a minimum of 4 weeks for removal of cellular debris). Figure 2e also illustrates that allodermis prepared by ethylene oxide sterilization (whether pre-treated with glycerol or not) retain the pliability of normal skin following 2 days soaking in phosphate buffered saline or in medium. This photograph shows a piece of de-epidermised acellular dermis draped over a needle to illustrate its pliability.

25

Effect of dehydration of skin prior to ethylene oxide sterilization

We were aware from previous studies that ethylene oxide sterilization appears to have the one disadvantage of causing some damage particularly to the reticular dermis. Hence, we explored a number of approaches to preventing

this damage while retaining the beneficial effects of ethylene oxide sterilization. We noted that with glycerol (which gives a slow dehydration of the skin), there was no apparent damage to the dermis. Accordingly, we explored initial dehydration of the skin with either glycerol or isopropanol prior to ethylene oxide sterilization. Table 2 shows the effects of the dehydration and sterilisation techniques on the physical strength of the dermal preparations. Five different donor preparations were tested and we examined the effect of glycerol and isopropanol and ethylene oxide on their own as well as in appropriate combinations. All results are expressed as a percentage of the values obtained for fresh and treated dermis. Tensile strength and strain were measured for all samples. Tensile strength or stress is the maximum load (kg/unit area mm²) which the sample could bear before breaking. Strain is the increase in length of the sample/unit of the original length of the sample until the sample broke. Of the various treatments, isopropanol did not affect the tensile strength whilst all others significantly increased it. Paradoxically isopropanol was the only treatment that significantly improved resistance to strain.

Although standard ethylene oxide treatment resulted in a high tensile strength, the percentage strain was less than normal skin reflecting the damage to the dermis that we had noted. This skin would break without much elongation reflecting the brittle nature of the skin. Glycerol treated skin showed higher tensile strength than isopropanol treated skin. Both glycerol and isopropanol pre-treatment with subsequent ethylene oxide sterilization resulted in good strength and good elongation compared to fresh dermis. On the basis of physical properties alone, both techniques of dehydration, isopropanol and glycerol, followed by ethylene oxide sterilization would give suitable dermal replacements.

However, in examining the appearance of the isopropanol/ethylene oxide dermis, we found the papillary dermis to have a more tightly packed conformation than in normal skin and there was some discolouration. Hence, this preparation was not carried forward for *in vitro* or *in vivo* composite production.

Following this investigation of physical properties it was decided to compare composites based on dermis produced using the standard ethylene oxide method or dermis produced using glycerol pre-treatment followed by ethylene oxide sterilization

Use of sterile allodermis in composite production

To compare and standardise results for composite performance both *in vitro* and *in vivo* (on nude mice), a scoring system was devised. As detailed in Table 3, this scoring system takes into account the nature of the dermis (whether it had normal architecture or not), the extent of fibroblast penetration into the dermis for those composites where fibroblasts were added, the nature of the keratinocyte layer, the nature of the keratin layer and the quality of the dermal epidermal junction. A maximal score of 15 was possible where both cell types were included in the composites with features resembling normal skin.

All composites were scored blind by 3 independent observers throughout the study and the median of these scores was taken.

We prepared composites both with and without fibroblasts and we examined the performance of these composites both *in vitro* and on the nude mice. All composites were cultured for 10 days at an air-liquid interface *in vitro* and either examined at this stage or 2 weeks post-grafting onto nude mice.

Figures 3, 4 and 5 show composites based on glycerol, ethylene oxide, and glycerol/ethylene oxide prepared dermis respectively. In each Figure, the *in vitro* and *in vivo* performance of the composites in the presence and absence of fibroblasts is depicted.

Table 4 compares the performance of composites based on ethylene oxide and glycerol/ethylene oxide sterilized skin. Four experiments are compared, each using skin from a separate donor dermis. (Table 5 compares the *in vivo* performance of the composites prepared using glycerol, ethylene oxide and glycerol/ethylene oxide sterilized composites on the nude mice.)

Figure 3 illustrates that glycerol sterilized dermis gave rise to composites which showed a poor epidermal attachment in the absence of fibroblasts once composites had undergone the shearing forces of cryosectioning. It is unlikely that such composites will provide secure epidermal attachment on a wound bed when subjected to any manipulation. Similarly, *in vivo*, composites prepared with fibroblasts performed relatively well on the nude mice (Figure 3c) but showed poor epidermal attachment on the mice in the absence of fibroblasts (Figure 3d).

In contrast, Figures 4 and 5 show that the composites prepared using ethylene oxide sterilization and sodium chloride removal of the epidermis performed much better than the glycerol based composites. Epidermal cells remained attached following cryosectioning both *in vitro* and *ex vivo*. Clearly composites could be prepared in the absence of fibroblasts and yet maintain secure epidermal attachment for at least 7 days in culture and after 2 weeks of grafting on the mouse.

Examining Table 4 for the semi-quantitative scoring of the ethylene oxide and glycerol/ethylene oxide composites *in vitro*, it was clear that inclusion of the glycerol dehydration step prior to ethylene oxide significantly improved the quality of the dermis. In contrast glycerol/ethylene oxide sterilized dermis
5 gave composites with flatter epidermal/dermal junctions than ethylene oxide sterilized composites. However, both protocols of ethylene oxide sterilization gave composites with a similar overall score *in vitro* (achieving an average of 11 out of a maximum of 15 points in comparison to normal skin). Thus, the addition of the glycerol dehydration step prior to the ethylene oxide
10 sterilization did not lead to any loss of composite quality, while slightly reducing the damage to dermis.

Table 5 summarizes the *in vivo* performance of composites prepared using glycerol, ethylene oxide and glycerol/ethylene oxide sterilized skin focussing
15 on the quality of the collagen IV basement membrane antigen layer in these composites both in the presence and absence of fibroblasts. As can be seen, the quality of the collagen IV in the glycerol composites was relatively poor irrespective of the presence of fibroblasts, whereas a good layer of collagen IV was seen in composites prepared with ethylene oxide or glycerol/ethylene
20 oxide. Thus it appears that the ethylene oxide sterilization protocol is clearly better than the glycerol based sterilization protocol for composite performance *in vivo*. Collagen IV staining was equally good in ethylene oxide sterilized composites and in those pre-treated with glycerol dehydration prior to ethylene oxide sterilization.

25

Also, we noted that keratinocytes expanded in culture could also be used to produce composites, which performed well (as will be discussed in the following section of the application).

Scaling up and speeding up of production of composites for clinical use

To address the issue of speeding up composite production, we then examined the use of keratinocytes expanded in culture in composite production. Our preliminary data in this area show that keratinocytes initially expanded in

5 culture will give reasonable composite performance both *in vitro* (data not shown) and *in vivo* (please see table 5) even in the absence of fibroblasts.

Table 1: Production of DED using ethylene oxide and 1M NaCl solution

Hours	De-Epidermisation			Acellularity			BM Retention		
	Skin	Skin	EOS	Skin	Skin	EOS	Skin	Skin	EOS
	4 ⁰ C	37 ⁰ C	37 ⁰ C	4 ⁰ C	37 ⁰ C	37 ⁰ C	4 ⁰ C	37 ⁰ C	37 ⁰ C
0	-	-	-	+++	+++	+	+++	+++	+++
2	-	-	-	+++	+++	+	+++	+++	+++
4	-	+	+	+++	+++	+	+++	+++	+++
8	-	+++	+++	+++	++	-	+++	++	++
16	-	+++	+++	+++	++	-	+++	+	+
24	+	+++	+++	+++	++	-	+++	+	+
48	++	+++	+++	++	+	-	++	+	+
72	+++	+++	+++	++	+	-	++	+	+
96	+++	+++	+++	++	+	-	++	+	+

5 EOS = Ethylene oxide sterilized skin

De-epidermisation:

+++ = complete de-epidermisation
continuous staining of BM

10 ++ = epidermis comes away in patches but is incomplete
staining of BM

+ = epidermis comes away in only a few places with difficulty
staining of BM

- = epidermis remains fully attached

Acellularity:

+++ = normal cellularity

++ = abnormal cells ± cellular debris

+ = cellular debris

- = no cellular material

BM Retention:

+++ = thick and

++ = less intense continuous

+ = discontinuous/patchy

- = no BM staining

Table 2: The influence of sterilisation protocols on the physical properties of dermis

<u>Dermal Preparation</u>	<u>% Tensile Strength/Stress</u> mean \pm SEM (p value)	<u>% Strain</u> mean \pm SEM (p value)
Fresh dermis	100	100
Glycerol	134 \pm 11 (< 0.005)	116 \pm 8
Isopropanol	97 \pm 13	151 \pm 9 (<0.05)
Freeze-dried and ethylene oxide	153 \pm 19 (<0.01)	85 \pm 10
Glycerol and ethylene oxide	175 \pm 27 (<0.01)	124 \pm 6
Isopropanol and ethylene oxide	179 \pm 33 (<0.05)	119 \pm 6

All preparations were compared to the equivalent untreated control dermis by

5 Student's t- test

Table 3: Skin Composite Scoring System

<u>Region</u>	<u>Findings/Description</u>	<u>Score</u>
Keratin Layer	Normal keratin	3
	Parakeratosis	2
	Thin, "nucleated" layer	1
	Non-existent	0
Keratinocyte Layer	Organised, thick (> 2-3 cells), differentiated	3
	Organised, thin (< 2-3 cells), continuous	2
	Monolayer, discontinuous	1
	Non-existent	0
Dermo-Epidermal Junction (DEJ)	Attached epidermis with rête ridges present	3
	Flat DEJ with attached epidermis	2
	Partial attachment of epidermis	1
	No attachment of epidermal layer	0
Dermis	Normal regular reticular and papillary regions	3
	Small gaps/holes, disorganised	2
	Large gaps/holes, disorganised	1
	Disintegrating	0
Fibroblasts	Good penetration and presence of cells	3
	Good penetration, but few cells present	2
	No penetration, cells at reticular surface	1
	No cells present	0

Table 4: Comparison of in vitro appearance of composites prepared using ethylene oxide and glycerol/ethylene oxide sterilised skin

	<u>Ethylene Oxide</u>	<u>Glycerol/Ethylene Oxide</u>	<u>Significance</u>
	mean \pm SEM	mean \pm SEM	p value
Keratin layer:			
K	1.8 \pm 0.06	2.0 \pm 0.20	n.s.
K & F	1.8 \pm 0.15	1.9 \pm 0.10	n.s.
All together	1.8 \pm 0.10	1.9 \pm 0.10	n.s.
Keratinocyte layer:			
K	2.1 \pm 0.13	2.0 \pm 0.04	n.s.
K & F	2.6 \pm 0.15	2.6 \pm 0.18	n.s.
All together	2.3 \pm 0.12	2.3 \pm 0.18	n.s.
DEJ:			
K	2.8 \pm 0.14	2.4 \pm 0.19	n.s.
K & F	2.8 \pm 0.10	2.0 \pm 0.20	<0.05
All together	2.8 \pm 0.09	2.2 \pm 0.15	<0.025
Dermal quality:			
No cells	1.75 \pm 0.24	2.4 \pm 0.15	n.s.
F	2.2 \pm 0.20	2.5 \pm 0.16	n.s.
K	2.2 \pm 0.15	2.7 \pm 0.09	<0.1
K & F	2.4 \pm 0.06	2.6 \pm 0.11	n.s.
All together	2.1 \pm 0.21	2.6 \pm 0.12	<0.005
Fibroblast penetration:			
F	2.1 \pm 0.4	1.5 \pm 0.4	n.s.
K & F	1.4 \pm 0.3	2.0 \pm 0.2	n.s.
All together	1.8 \pm 0.3	1.8 \pm 0.2	n.s.
Total score for composites:			
K	8.8 \pm 0.36	9.1 \pm 0.36	n.s.
K & F	11.0 \pm 0.70	11.1 \pm 0.36	n.s.

n.s.=notsignificant

Table 5: Influence of sterilization methodology on performance of composites on nude mice

<u>Sterilization protocol</u>	<u>Presence of collagen IV in composite</u>		<u>Significance</u>	<u>All together (n)</u>
	No fibroblasts (n)	Fibroblasts(n)		
<u>Glycerol</u>	1 ± 0.3 (7)	0.9 ± 0.5 (7)	n.s.	0.9 ± 0.25 (14)
<u>Ethylene oxide a</u>	2.3 ± 0.1(15)	2.3 ± 0.2 (15)	n.s.	2.3 ± 0.1 (30)***
<u>Ethylene oxide b</u>	2.0 ± 0.3 (5)	2.0 ± 0.4 (6)	n.s.	2.0 ± 0.2 (11)**
<u>Glycerol/ Ethylene oxide</u>	2.0 ± 0 (5)	2.5 ± 0.2 (4)	n.s.	2.2 ± 0.13 (9)**

- 5 Scoring for collagen IV: 0 = none, 1 = patchy, 2= thin and continuous, 3 = thick and continuous

All composites were cultured for 10 days *in vitro* with or without the addition of fibroblasts as indicated and then grafted onto nude mice and assessed at 2 weeks. Collagen IV was scored blind on an arbitrary scale of 0-3, where 3 was based on the findings in normal skin. Additionally, keratinocytes initially expanded in culture were also used on ethylene oxide sterilised dermis (indicated by ethylene oxide (b), where (a) denotes freshly isolated cells).

- 15 The influence of fibroblasts on composite performance was assessed using Student's paired t-test, as each mouse received 2 composites, one with and one without fibroblasts. When it was ascertained that fibroblast addition did not influence collagen IV presence, then the data for composites with and without fibroblasts were combined. Student's non-paired t-test was used to compare between groups and values differing significantly from glycerol sterilized composites are indicated as **p< 0.005 or ***p< 0.001.

DISCUSSION

The aim of this study was to progress the development of a human skin replacement based on a sterile human allodermis with cultured autologous keratinocytes for clinical use. We report methodologies which allow composites to be produced and ready for grafting on a small scale within 2 weeks. Confirmation of the *in vivo* performance of these composites was achieved using the nude mouse model. Many studies of epidermal/dermal composites, based either on human allodermis or artificial dermis show that composites can be produced to an experimental level and can perform well on nude mice (please see refs 1-12).

To the best of our knowledge, this is the first study to demonstrate that such composites can be successfully produced using a sterilised human dermis. We suggest that this will prove a significant advance in the clinical usefulness of this material.

The main issues we considered in advancing the development of composites from the laboratory to clinical use were how sterilisation of the skin might affect the morphology of the skin and the residual basement membrane antigens which we had previously established were essential for secure keratinocyte attachment in vitro (Ralston et al 1998)(16). In the absence of basement membrane antigens, keratinocytes will attach initially but (from our experience) this attachment deteriorates with time in vitro. The most important single factor associated with de-epidermisation is the retention of as much basement membrane as possible at the dermo-epidermal junction. Other groups have reported the importance of the basement membrane in determining secure and preferential attachment of keratinocytes (Murray et al 1979 and Terranova et al 1980)(26,27).

Our previous work using glycerol sterilised dermis has shown that even when basement membrane antigens are present initially, there is a requirement for continued fibroblasts presence in vitro to avoid deterioration of this basement
5 membrane zone (Ralston et al 1998)(16).

Focusing on the requirement for good basement membrane retention, our objective was firstly to establish a sterilization protocol which allowed retention of a good basement membrane zone following removal of the
10 epithelium and removal of donor fibroblasts. This acellular allodermis had to be suitable for production of composites with a secure epidermal/dermal junction when assembled in the laboratory and when assessed after two weeks of grafting on nude mice. Our second objective related to speeding up composite production and here we wished to see if we could provide such
15 epidermal/dermal attachment without the need for donor fibroblasts. Following on from this, we addressed the problem of scaling up the provision of epidermal/dermal composites for clinical use, making use of this allodermis using keratinocytes either expanded in culture, or freshly isolated and seeded at a lower density to go further.

20

We explored a combination of sterilisation and de-epidermisation techniques, which would allow good retention of basement membrane antigens and good retention of dermal morphology. We found that optimisation of conditions for removal of the epidermis (using 1M sodium chloride at 37°C for 8 hours) and
25 introducing a preparative gentle dehydration step prior to ethylene oxide sterilisation of skin gave a de-epidermised acellular sterile dermis with good dermal morphology and good retention of basement membrane antigens. This material would also be suitable for use as a dermal replacement on its own.

We also found that it performed well as a dermal substrate for the production of reconstructed skin using keratinocytes both with and without fibroblasts.

With respect to dermal morphology, we have previously shown that glycerol
5 sterilisation of skin is almost certainly based on the hygroscopic properties of glycerol which allow it to slowly dehydrate the skin providing the basis of its bacteriacidal and viricidal activity (Marshall et al 1995)(19). Thus we have shown that exposure to 98% glycerol for 3 weeks at room temperature is sufficient to prevent infectivity of herpes simplex and polioviruses. However,
10 although glycerol has been shown to be effective in the elimination of viruses, bacteria and fungi (Marshall et al 1995, Basile et al 1982, Van Baare et al 1994)(19,24,25) it is not a standard method of sterilisation. Dermis produced by this method maintains good histological morphology and remains pliable with good handling properties and retains expression of some basement
15 membrane antigens. Following on from glycerol sterilisation of dermis, we then explored ethylene oxide sterilisation of dermis and showed that successful composites could be produced using ethylene oxide sterilised skin. Ethylene oxide sterilization is a universally recognised method of sterilization used for surgical equipment (Brigden et al 1980)(28). It is readily available
20 and is a widely used sterilization process, being present in the majority of hospitals.

However, there was a variable degree of ethylene oxide induced damage to the dermis which was most evident in the reticular dermis. Using a standard
25 protocol of ethylene oxide sterilisation ie. lyophilisation of material prior to ethylene oxide sterilisation, we were unable to prevent this dermal damage (Ghosh et al 1997)(23). However, we accept that other protocols of ethylene oxide sterilisation do exist which may result in less damage although this is not evident from the literature. The current study shows that by combining the

hygroscopic properties of glycerol to achieve a more gentle dehydration over the first 2 days with ethylene oxide sterilisation, we achieve sterilised dermis with very little ethylene oxide induced damage to the reticular dermis. A similar result could be achieved using isopropanol as a preparative dehydration step but we noted that there was some compaction of the papillary dermis with isopropanol and some discolouration of the skin. We did not explore isopropanol/ethylene oxide sterilised skin further in composite production in this study.

10 The issue of remodelling of the basement membrane zone is clearly different *in vitro* and *in vivo*. *In vivo*, basement membrane formation has been demonstrated as early as 6 days on mice grafted with bovine collagen I lattice preseeded with fibroblasts in cultured keratinocytes (Hansbrough et al 1994)(5). However, *in vitro*, we found that with glycerol sterilised dermis, 15 both fibroblasts and keratinocytes were required for retention of (or remodelling of) the basement membrane zone when composites were maintained in culture throughout 3 weeks. Other studies have found that remodelling of the basement membrane zone is greatly enhanced if both fibroblasts and keratinocytes are present in composites than only one cell type 20 (Konig et al 1991 and Ralston et al 1997)(30,16). Up to 10 days, keratinocytes appeared to attach well to composites in the absence of fibroblasts. However, sectioning at this point revealed not only poor epithelial cell attachment to the underlying dermis but also very poor organisation of the epidermal layer. This is consistent with a previous report based on collagen 25 sponge (Maruguchi et al 1994)(32). Thus, in providing a one-stage material, we decided that it was important to address the issue of secure epidermal cell attachment to the dermis as any grafted material would be expected to undergo some degree of shear forces following grafting onto the patient.

The role of fibroblasts in maintaining/remodelling the basement membrane is not surprising. However, we asked whether good keratinocyte attachment could be achieved with a good basement membrane in the absence of fibroblasts for one practical reason. While it is feasible to culture large numbers of keratinocytes within 2 - 3 weeks, we find it takes much longer to obtain the necessary expansion of autologous fibroblasts for composite production. Thus, if one wanted to use autologous keratinocytes and fibroblasts in composite production, this would have implications for the speed with which reasonable areas of composite material could be produced.

Other studies have shown culture and application of composites without fibroblasts onto nude mice (Medalie et al 1996 and Demarches et al 1992)(11,12) to be successful, in both cases using a non-sterile human acellular de-epidermised dermis. Thus, the question appeared to be that if a good basement membrane region were present, could composites be prepared for clinical use in the absence of fibroblasts?

The data presented in this study clearly shows that by 2 weeks there is infiltration of host (mouse) fibroblasts into the composites such that there was no significant advantage to adding human fibroblasts to the composites prior to grafting. Composites engrafted successfully and were stable with secure epidermal/dermal junctions, which resisted the shear forces of sectioning at 2 weeks.

We also began to address the final issue of scaling up and speeding up this methodology in this study to make such composites clinically useful. We confirmed that keratinocytes expanded in culture can be used to produce successful composites on nude mice and, further, that there are two options to the expansion either using the application of cultured epithelial autografts onto the sterilised dermal preparations or using passaged keratinocyte suspensions.

Cultured keratinocytes have been used in composites on nude mice (please see refs 3-6 and 8-12) and have been used in conjunction with a human allodermis (Medalie et al 1996 and Demanches et al 1992)(11,12). Work is now continuing in this laboratory to scale up and speed up the production of
5 such composites based on this information.

In conclusion, this study shows that by paying attention to the details of sterilisation of the dermis and removal of the epidermis, a sterilised human acellular DED can be produced with essentially normal dermal morphology
10 and retention of basement membrane antigens. This acellular DED would be appropriate for clinical use as a dermal replacement material.

We have also shown that it can be used to produce composites which perform well on nude mice at 2 weeks and that there is the potential to speed up and
15 scale up the production of this material as keratinocytes initially expanded in culture also perform well on this dermis.

Expediting and expanding composite production should now be feasible for clinical use. The sterilised allodermis can be stored on the shelf requiring only
20 the addition of the expanded patient keratinocytes. We suggest that this will represent a significant advance on other methodologies currently available for treating patients with extensive full thickness skin loss.

REFERENCES

1. Manning D., Reed N. & Shaffer C., (1973). Maintenance of skin xenografts of widely divergent phylogenetic origin on congenitally athymic (nude) mice. J Exp. Med., 138, 488-494.
2. Demarchez M., Desbas C. & Prunieras M., (1985). Wound healing of human skin transplanted on to the nude mouse. Brit J Dermatol 113, Supplement 28, 177-182.
3. Bosca A.R., Tinois E., Faure M., Kanitakis J., Roche P. & Thivolet J., (1988). Epithelial Differentiation of human skin equivalents after grafting onto nude mice. J Invest Dermatol Vol. 91: 136-141, 1988.
4. Cooper M.L & Hansbrough J.F., (1991). Use of a composite skin graft composed of cultured human keratinocytes and fibroblasts and a collagen-GAG matrix to cover full-thickness wounds on athymic mice. Surgery 109: 198-206.
5. Hansbrough J.F., Morgan J., Greenleaf G., Parikh M., Nolte C. & Wilkins L., (1994). Evaluation of Graftskin Composite Grafts on Full-Thickness Wounds on Athymic Mice. J Burn Care Rehabil Vol. 15, No. 4, 346-353.
6. Boyce S.T., Foreman T.J., English K.B., Stayner N., Cooper M.L., Sakabu S. & Hansbrough J.F., (1991). Skin wound closure in athymic mice with cultured human cells, biopolymers, and growth factors. Surgery, 110 (5): 866-876.

7. Hansbrough J.F., Cooper M.L., Cohen R., Spielvogel R.L., Greenleaf G., Bartel R.L. & Naughton G., (1992). Evaluation of a biodegradable matrix containing cultured human fibroblasts as a dermal replacement beneath meshed skin grafts on athymic mice. *Surgery* 111: 438-446.
8. Boyce S.T., Medrano E.E., Abdel-Malek Z., Supp A.P., Dodick J.A.M., Nordlund J.J. & Warden G.D., (1993). Pigmentation and Inhibition of Wound Contraction by Cultured Skin Substitutes with Adult Melanocytes After Transplantation to Athymic Mice. *J Invest Dermatol* Vol.100, No. 4, 360-365.
9. Cooper M.L., Andree C., Hansbrough J.F., Zapata-Sirvent R.L., & Spielvogel R.L., (1993). Direct Comparison of a Cultured Composite Skin Substitute Containing Human Keratinocytes and Fibroblasts to an Epidermal Sheet Graft Containing Human Keratinocytes on Athymic Mice. *J Invest Dermatol* Vol.101, No. 6, 811-819.
10. Boyce S.T., Supp A.P., Harriger D., Greenhalgh D.G. & Warden G.D., (1995). Topical Nutrients Promote Engraftment and Inhibit Wound Contraction of Cultured Skin Substitutes in Athymic Mice. *J Invest Dermatol* Vol.104, No. 3, 345-349.
11. Medalie D.A., Erning S.A., Tompkins R.G., Yarmush M.L., Krueger G.G. & Morgan J.R., (1996). Evaluation of Human Skin Reconstituted from Composite Grafts of Cultured Keratinocytes and Human Acellular Dermis Transplanted to Athymic Mice. *J Invest Dermatol* Vol.107, No. 1, 121-127.

12. Demarchez M, Hartmann DJ, Regnier M, Asselineau D (1992). The role of fibroblasts in dermal vascularisation and remodelling of reconstructed human skin after transplantation onto the nude mouse. Transplantation 54,317-326.
- 5
13. Compton C, Gill JM, Bradford DA, Regauer S, Gallico GG, O'Connor NE, (1989). Skin regenerated from cultured epithelial autografts on full-thickness wounds from 6 days to 5 years after grafting: a light, electron microscopic and immunohistochemical study. Lab. Invest , 60, 600-612.
- 10
14. Desai MH, Mlakar JM, McCauley RL, Abdullah KM, Rutan RL, Wymack JP et al., (1992). Lack of long term durability of cultured keratinocyte burn-wound coverage: a case report. J Burn Care Rehab. 13: 174-180.
- 15
15. Nanchahal J, Ward CM, (1992). New grafts for old? A review of alternatives to autologous skin. Brit J Plast Surg, 45:354-363.
- 20
16. Ralston DR, Layton C, Dalley AJ, Boyce SG, Freedlander E, MacNeil S, (1998). Investigation of basement membrane formation in vitro in human epidermal/dermal skin equivalents.
- 25
17. Goberdhan NJ, Dawson Ra, Freedlander E, MacNeil S, (1993). A calmodulin-like protein as an extracellular mitogen for the keratinocyte. Br J Dermatol, 129:678-688.
18. Rheinwald JG, Green H (1975). Serial cultivation of strains of epidermal keratinocytes: the formation of keratinising colonies for single cells. Cell, 6: 331-344.

19. Marshall L, Ghosh MM, Boyce S, Freedlander E, Kudesia G, MacNeil S, (1995). The effect of glycerol on intracellular viruses; implications for the clinical use of glycerol preserved cadaver skin. *Burns*, 21:356-361.
- 5
20. Krejci NC, Cuono CB, Langdon RC, McGuire J,(1991). In vitro reconstitution of skin: fibroblasts facilitate keratinocyte growth and differentiation on acellular reticular dermis. *J Invest Dermatol* 97, 843-848.
- 10
21. Woodley, D., Sauder, D., Talley, M. J., Silver, M., Grotendorst, G., and Qwarnstrom, E.(1983). Localisation of basement membrane components after dermal-epidermal junction separation. *J Invest Dermatol* 81, 149-153.
- 15
22. Moore JC, Ray AK, and Shakespeare, P. ,G.(1993). Surface ultrastructure of human dermis and wounds. *Br J Plast Surg* 46, 460-465.
- 20
23. Ghosh MM, Boyce S, Layton C, Freedlander E, MacNeil S, (1997). A comparison of methodologies for the preparation of human epidermal-dermal composites. *Ann Plast Surg* 39, 390-404.
- 25
24. Basile ARD (1982). A comparative study of glycerinised and lyophilised porcine skin in dressings for third degree burns. *Plast Reconstr Surg*. 69: 969-972.
- 25
25. Van Baare J, Buitenwerf J, Hoekstra MJ, du Pont JS, (1994). Virucidal effect of glycerol as used in donor skin preservation. *Burns* 20: S77-S80.

26. Murray JC, Stingl G, Kleinman HK, Martin GR, Katz SI, (1979). Epidermal cells adhere preferentially to Type IV (basement membrane) collagen. *J Cell Biol.* 80:197-202.
- 5 27. Terranova VP, Rohrbach DH, Martin GR, (1980). Role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen *Cell*, 22:719-726.
28. Brigden R, (1980). *Operating Theatre Technique*. 4th Ed. Edinburgh: Churchill Livingstone.
- 10
29. Kearney JN, Franklin UC, Aguirregocoa V, Holland KT, (1989). Evaluation of ethylene oxide sterilisation of tissue implants. *J Hosp Infection*, 13: 71-80.
- 15
30. Konig A, Bruckner-Tuderman L, (1991). Epithelial-mesenchymal interactions enhance expression of collagen VII in vitro. *J Invest Dermatol*, 96,803-808.
- 20 31. Ralston DR, Layton C, Dalley AJ, Boyce SG, Freedlander E, MacNeil S. (1997). Keratinocytes contract human dermal extracellular matrix and reduce soluble fibronectin production by fibroblasts in a skin composite model. *Br J Plast Surg*.
- 25 32. Maruguchi T, Maruguchi Y, Suzuki S, Matsuda K, Toda K, Isshiki N, (1994). A new skin equivalent: keratinocytes proliferated and differentiated on collagen sponge containing fibroblasts. *Plast Reconst Surg*, 93, 537-544.

33. Herzog SR, Meyer A, Woodley D, Peterson HD, (1988). Wound coverage with cultured autologous keratinocytes: use after burn wound excision, including biopsy followup. J Trauma, 28: 195-198.
- 5 34. Woodley DT, Peterson HD, Herzog SR, Stricklin GP, Burgeson RE, Briggaman RE, Cronce DJ, O'Keefe EJ, (1988). Burn wounds resurfaced by cultured epidermal autografts show abnormal reconstitution of anchoring fibrils. JAMA, 259: 2566-2571.
- 10 35. Green H, Kehinde O, Thomas J, (1979). Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. Proc Natl Acad Sci. USA, 76:5665-5668.
- 15 36. Banks-Schlegel S & Green H (1980). Formation of epidermis by serially cultivated human epidermal cells transplanted as an epithelium to athymic mice. Transplantation, 29: 308-313.
37. Cuono et al. Proc.Natl.Acad.Sci. USA, 1987;80;826-835.

CLAIMS

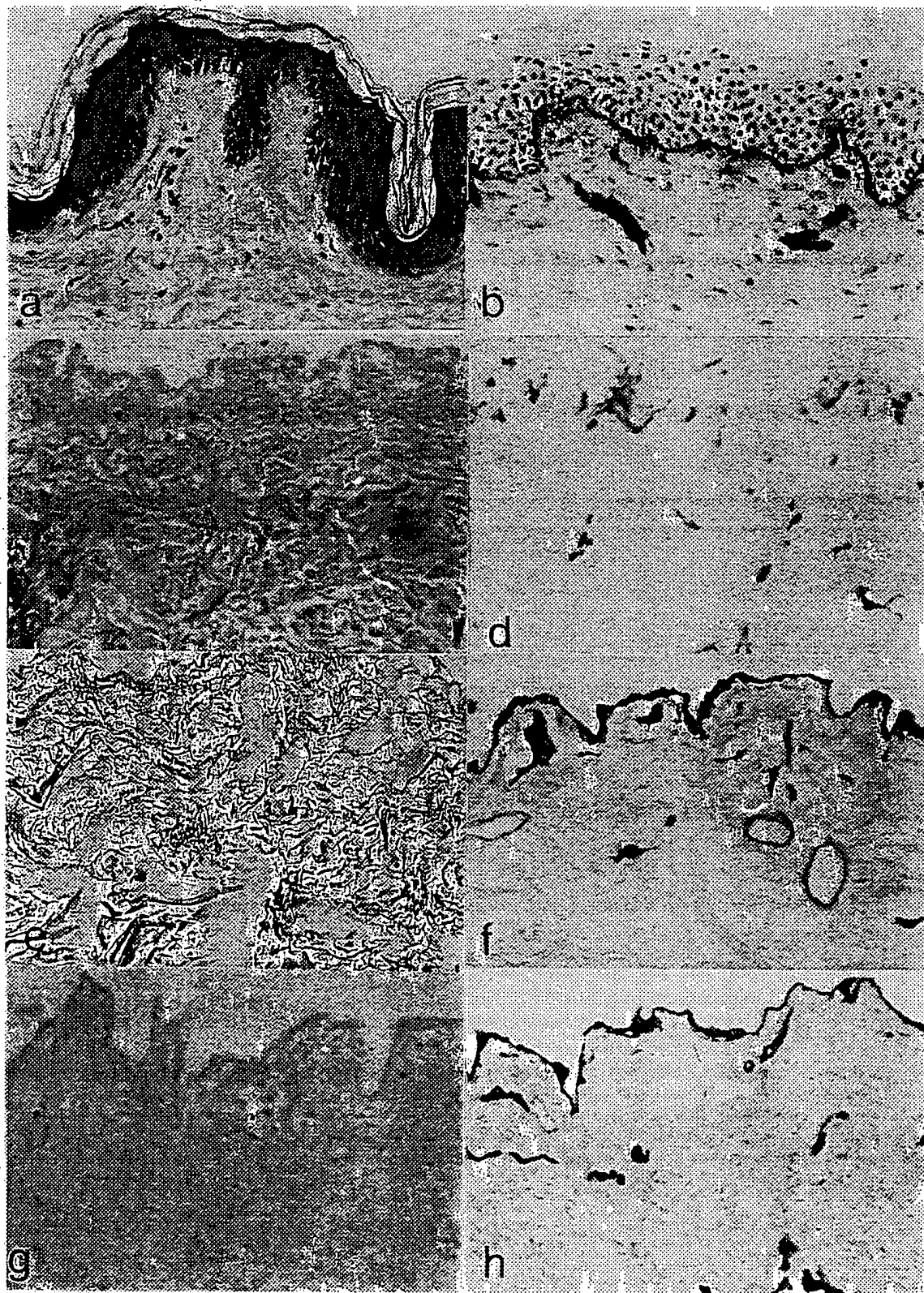
1. A method for providing a collagen based tissue for use in tissue replacement comprising:
 - 5 (i) harvesting a collagen based tissue from a human or animal;
 - (ii) removing the cellular elements from said collagen based tissue;
 - (iii) dehydrating said collagen based tissue in the presence of a sterilising agent; and
 - 10 (iv) removing said dehydrated and sterilised collagen based tissue from said dehydrating and sterilising agents.
2. A method according to Claim 1 characterised in that said collagen based tissue is selected from at least one of gastric tissue; venous vessels; arterial vessels; heart valves; bone; oral mucosa; or skin.
- 15 3. A method according to Claim 1 or 2 characterised in that said collagen based tissue is derived from human skin.
4. A method according to any of Claims 1 – 3 characterised in that said human skin is an allograft.
- 20 5. A method according to any of Claims 1 - 4 characterised in that said dehydration of collagen based tissue is through incubation with a liquid dehydration agent.
- 25 6. A method according to Claim 5 characterised in that said dehydration of collagen based tissue is through a sequential and incremental increase in the concentration of said dehydration agent.
- 30 7. A method according to Claims 1-6 characterised in that said dehydration agent concentration is increased from at least 50% to at least 98%.

8. A method according to Claims 6 or 7 characterised in that said increase in said dehydration agent concentration is in increments of at least 10%.
- 5 9. A method according to Claims 5 - 8 characterised in that said dehydration agent is glycerol.
10. A method according to Claims 5 - 8 characterised in that said dehydration agent is isopropanol.
- 10 11. A method according to Claims 1-10 characterised in that said sterilising agent is a fluid.
12. A method according to Claim 11 characterised in that said sterilising agent is a liquid.
- 15 13. A method according to Claim 11 characterised in that said sterilising agent is gaseous.
- 20 14. A method according to Claim 13 characterised in that said sterilising agent is gaseous ethylene oxide.
15. A method according to Claim 14 characterised in that said ethylene oxide is provided at 15% ethylene oxide, carbon dioxide is provided at 85%, at an atmospheric pressure of at least 5.5 atmospheres at between 45-55°C for at least 30 minutes.
- 25 16. A composition comprising a collagen based tissue produced by the method to any of Claims 1 - 15.

30

17. A composition according to Claim 16 characterised in that said tissue is a human skin composite.
18. A composition according to Claims 18 or 19 for use as a therapeutic composition.
19. A product comprising a therapeutic composition according to Claim 18 and provided with storage means which facilitate and/or maintain the natural structural conformation of the skin composite.
20. A method according to Claims 1-15 characterised in that said method is for the preparation of collagen based tissues for use in cosmetic reconstructive surgery.
21. A method of treatment comprising a collagen based tissue for use in tissue replacement therapy comprising;
- i) preparation of a collagen based tissue according to any of Claims 1 – 15;
 - ii) surgical insertion of said collagen based tissue to a patient to be treated; and, optionally
 - iii) monitoring the status of said inserted collagen based tissue.
22. A method according to Claim 21 characterised in that said collagen based tissue is human skin.
23. An isolated collagen based tissue characterised in that said tissue substantially retains its natural conformation.

Figure 1



SUBSTITUTE SHEET (RULE 26)

FIGURE 2

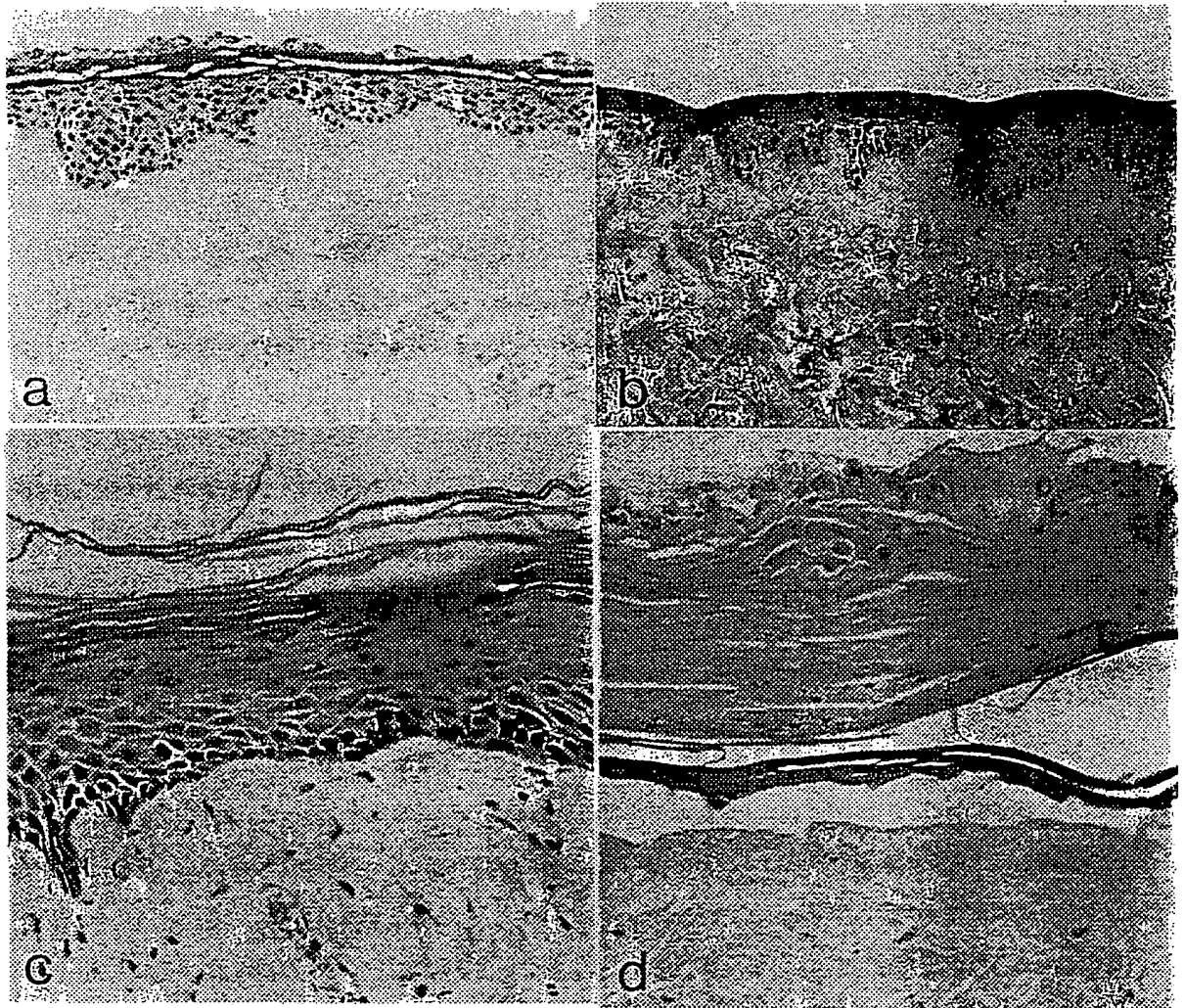
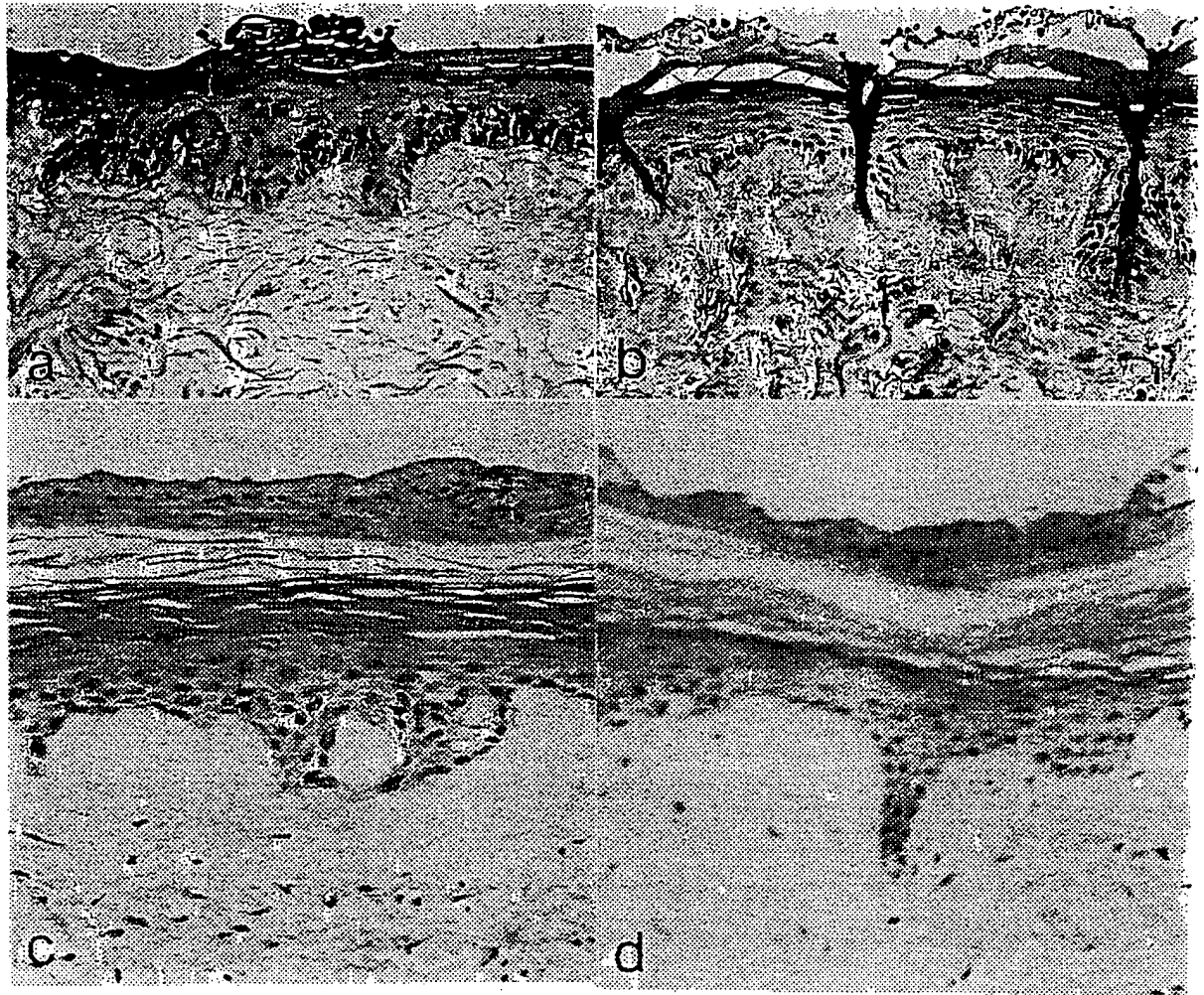


FIGURE 3



SUBSTITUTE SHEET (RULE 26)

FIGURE 4

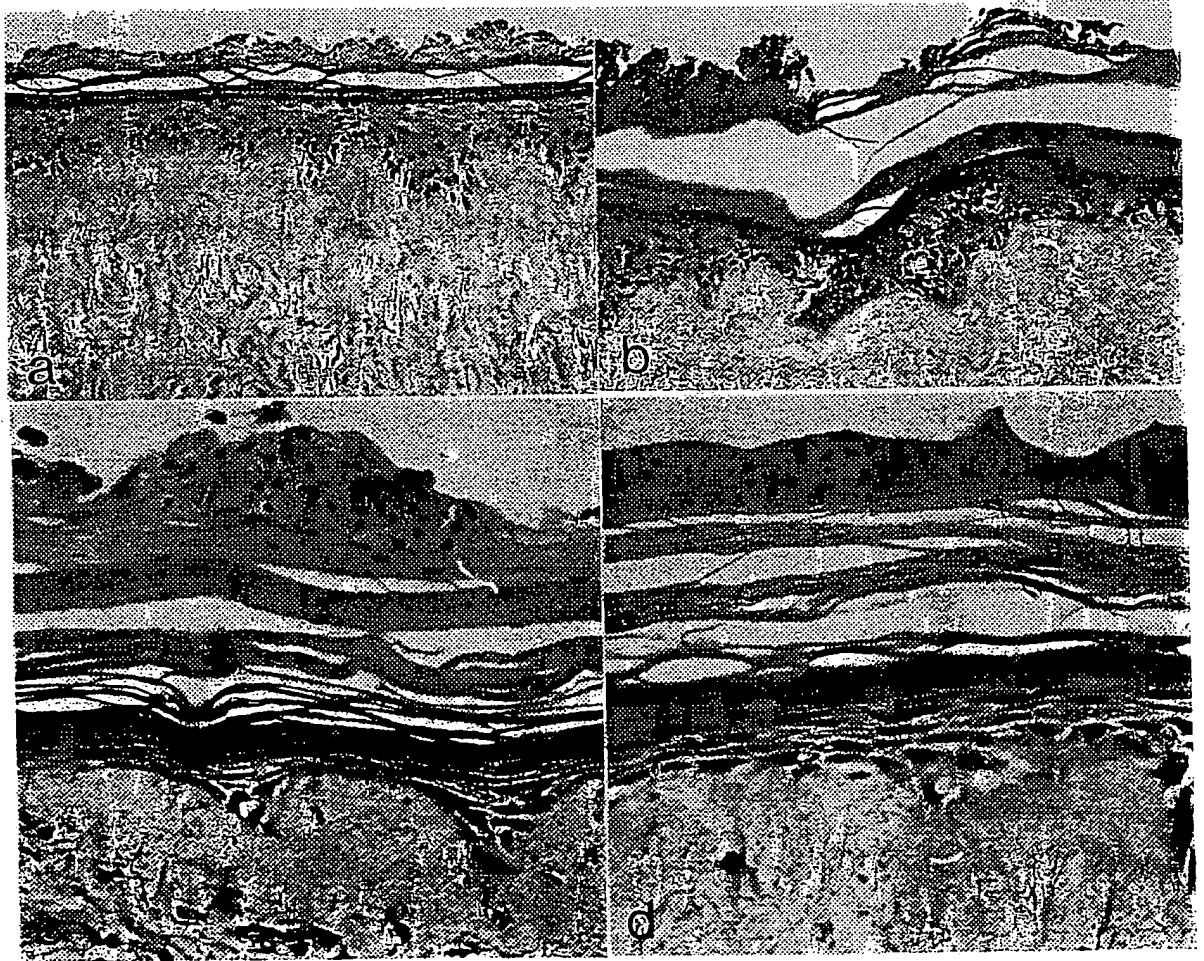
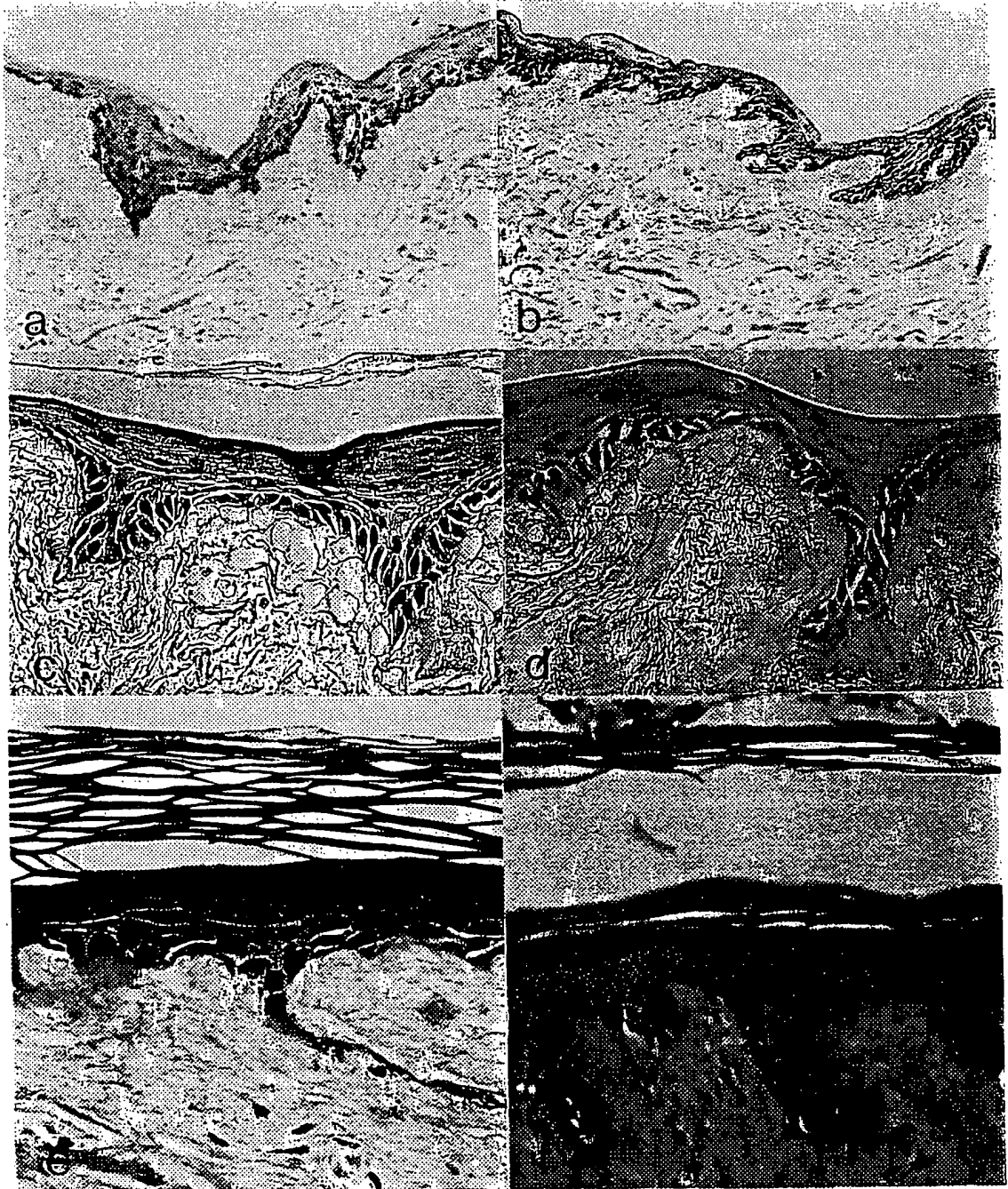


FIGURE 5



SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/03889

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61L27/36 A61L27/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61L A61F C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CHAKRABARTY K.H., DAWSON R.A., HARRIS P.: "Development of autologous human dermal-epidermal composites based on sterilized human allodermis for clinical use" BRITISH JOURNAL OF DERMATOLOGY, vol. 141, 1999, pages 811-823, XP000878676 abstract page 812, left-hand column, paragraph 2 -page 814, left-hand column, paragraph 3 -/-	1-23

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document relating to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

11 February 2000

Date of mailing of the international search report

23/02/2000

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Menidjel, R

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 99/03889

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GHOSH M.M., BOYCE S., LAYTON C., FREEDLANDER E., MACNEIL S.: "A comparison of Methodologies for the preparation of Human Epidermal-Dermal composites" ANNALS OF PLASTIC SURGERY, vol. 39, no. 4, 1997, pages 390-404, XP000878553 cited in the application abstract page 391, left-hand column, paragraph 2 -page 392, left-hand column, paragraph 3 page 392, right-hand column, paragraph 2 -page 393, left-hand column, paragraph 2	1-9, 11, 13-23
X	WO 98 07452 A (SULZER VASCUTEK LIMITED ;WALKER DONALD FRANCIS (GB)) 26 February 1998 (1998-02-26) page 2, line 2 -page 3, line 27 page 4, line 20 - line 31 example 1	1,5-9, 11,13-15
X	US 5 263 983 A (KOIDE MIKIO ET AL) 23 November 1993 (1993-11-23) column 4, line 18 - line 65 column 6, line 58 -column 7, line 54 examples 1,5	1-3, 16-23
A	DE 44 25 776 A (SCHIMMACK BRITTA) 25 January 1996 (1996-01-25) abstract column 1, line 3 - line 56	1,2,5,9, 16,20, 21,23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 99/03889

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 21,22 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box 1.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal
body by therapy

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 21,22 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/03889

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9807452 A	26-02-1998	AU 3950297 A	06-03-1998
US 5263983 A	23-11-1993	JP 1230366 A	13-09-1989
		JP 6022579 B	30-03-1994
		JP 2034171 A	05-02-1990
		JP 1890522 C	07-12-1994
		JP 2034165 A	05-02-1990
		JP 6018583 B	16-03-1994
		JP 2071748 A	12-03-1990
		JP 2645098 B	25-08-1997
		AU 632273 B	24-12-1992
		AU 3212689 A	05-10-1989
		DE 68915540 D	30-06-1994
		DE 68915540 T	13-10-1994
		EP 0403650 A	27-12-1990
		WO 8908465 A	21-09-1989
		AU 632471 B	07-01-1993
		AU 3212589 A	05-10-1989
		DE 68909933 D	18-11-1993
		DE 68909933 T	03-03-1994
		EP 0411124 A	06-02-1991
		WO 8908466 A	21-09-1989
		US 5350583 A	27-09-1994
DE 4425776 A	25-01-1996	NONE	

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